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14. ABSTRACT A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including scoliosis and pseudoarthrosis, which are compounded by osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. The cell types and pathway by which neurofibromin haploinsufficiency (Nf1 +/-) leads to dysregulation of bone remodeling and healing are unknown. The aim of this study is to identify the cell types expressing Nf1 in normal bone cell physiology and fracture healing and to test the Nf1 requirement in vitro. We have employed in vitro and in vivo models to test the effects of neurofibromin deficiency in bone-forming osteoblasts and in bone-resorbing osteoblast cells. In addition, we have established a collection of bone tissue samples from NF1 patients to characterize the bone, tissue, and cells of NF1 bone.

15. SUBJECT TERMS
Neurofibromatosis, NF1, fracture, osteoporosis, orthopedic

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INTRODUCTION

A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including scoliosis, pseudoarthrosis, and sphenoid bone dysplasia, which are compounded by osteoporosis and poor bone healing. Corrective orthopedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. The cell types and pathway by which neurofibromin haploinsufficiency (Nf1+/–) leads to dysregulation of bone remodeling and healing are unknown. Recently pseudoarthrosis was reported to be associated with loss of heterozygosity (LOH) at the NF1 locus, implying that loss of both NF1 alleles in bone results in pseudoarthrosis (1).

Normal bone development, bone healing, and bone metabolism require the concerted actions of bone forming osteoblasts and bone resorbing osteoclasts. Osteoblast differentiation is regulated by intrinsic and local factors while the formation and function of osteoclasts is regulated primarily by osteoblasts through their expression of pro- and anti-osteoclastic factors.

We hypothesize that neurofibromin haploinsufficiency results in dysregulated bone cell differentiation and/or function. Dysregulation occurs through direct effects on osteoblasts or their precursors, through osteoblast-mediated osteoclast formation, or alternatively, through direct effects in the osteoclast itself.

Objectives:

Aim 1. This aim will test the hypothesis that the abnormal bone remodeling associated with NF1 is related to dysregulated osteoblast and/or osteoclast differentiation and activity. We will Identify the target cells (osteoblasts or osteoclasts) mediating dysregulated bone cell formation and function in Nf1+/- mouse cells in culture and assess the role of NF1 in normal bone cell function.

Aim 2. This aim will test the hypothesis that the neurofibromin gene plays an essential role in fracture healing. We will characterize neurofibromin function in bone cells in a fracture repair model.

Aim 3. This aim will test the hypothesis that the tissue and cellular pattern of distribution and functional role of the Nf1 gene identified in the haploinsufficient mouse model will be predictive of the role and distribution of this gene in patients with NF1. We will characterize bone cells in bone samples from Orthopedic NF1 patients.

Beyond providing insights into the molecular pathogenesis of skeletal disease and bone resorption in NF1, this study will determine if current methods of pharmacological intervention can modulate NF1 bone cell differentiation and function in cell culture. We believe that valuable information can be gained using well-established models of bone cell formation and function in NF1 and that the results from the proposed studies may quickly be translated into therapies for orthopedic NF1 patients.

BODY

This report details progress toward the specific aims of this project from December 15th 2005 to December 14th 2008, with some modification of the approach. While we have made progress, over the course of the project unforeseen circumstances have impacted our timeline. Where possible, our approach was modified to enable progress despite delays in outlined tasks. The first of which was a significant delay in obtaining and establishing a mouse breeding colony as described below under *Task 1*. An alternate animal model was pursued which incurred unrelated further delay. *Task 1* is complete; however this has impacted other tasks. A second delay was encountered in the summer and fall of 2008 when the Hospital moved our laboratory from the second floor of the Harvard Institutes of Medicine building to the Research-North building. With this move, the New England Baptist Bone and Joint Institute, to which our laboratory group belonged, was disbanded. We joined the Department of Orthopedic Surgery in the Orthopedic Biomechanics Laboratory (OBL). Moving of the laboratory was disruptive to ongoing studies, personnel, and animals. In addition, the OBL has sufficient laboratory, office, and animal space for our group and for these studies however, OBL was not equipped for cellular and molecular biological studies. Therefore, a large amount of equipment had to be located or replaced, which has led to additional delays. At this point, we are still not completely settled, however most major equipment has been procured and experiments are in progress.

Task 1. **Establish an Nf1+/- mouse breeding colony:** We experienced significant delay in establishing a working breeding colony of the Nf1 +/- mice as described in *Task 1*. Nf1 +/- mice were originally ordered from Jackson labs to be recovered from cryopreservation, a procedure which normally takes over seven months. After a considerable delay we discovered that the order had not been filled. We have taken several approaches to circumvent this limitation consistent with **Aim 1**. In collaboration with Dr. Florent Elefteriou of Vanderbilt University, we have imported Col1-Cre, Nf1 flox/flox mice. In these animals the loxP site flanked Nf1 (flox/flox) gene is specifically deleted in any cell lineage expressing the Cre recombinase, but left intact in every other cell. Dr. Elefteriou has inserted a collagen type 1 promoter driven Cre transgene in these animals. The resulting Nf1flox/flox;col1Cre animals are deleted of both copies of Nf1 in the osteoblast lineage resulting in a bone phenotype (Nf1ob/ob mice) (2). In addition to providing more animals quicker, for *Tasks* 1-4, this approach may be more relevant to skeletal clinical conditions in NF1 since loss of heterozygosity in NF1 has been implicated in the pathogenesis of pseudoarthrosis of the tibia (1). Health reports from the Vanderbilt mouse colony indicated exposure to mouse parvo virus (MPV) and therefore imported animals underwent quarantining off-site at Charles River Labs, followed by testing for pathogens. Animals were imported into the BIDMC facility following the quarantine period and two, clean, consecutive monthly health reports, consistent with BIDMC Animal Research Facility animal import regulations. The colony is comprised of, and maintained as, col1-Cre on an Nf1 wild-type background (col1-Cre), Nf1flox/flox in the absence of col1-Cre (Nf1flox/flox), and col1-Cre Nf1flox/flox (Nf1ob/ob). PCR genotyping of individuals representative of each line is depicted in **figure 1 (appended item 3)**. Animals heterozygous for Nf1 in osteoblasts (col1-Cre Nf1+/flox) are generated in a single mating (the F1 generation of an Nf1ob/ob X col1-Cre). Homozygous knockout for Nf1 (Nf1-/-) can also be induced in osteoclast lineage cells in the Nf1flox/flox animals by crossing with the cathepsin-k promoter driven Cre, the MxCre transgene (available from Jackson Labs) (3), by transduction with adneo-Cre (available from the Harvard Gene Therapy Initiative), or by protein transduction with TAT-Cre, an approach which we have experience with (4, 5).

Task 2. **Characterize osteoblast differentiation and function in Nf1+/- mouse:** We used immunohistochemical staining to identify cells expressing neurofibromin in mouse bone tissue *in vivo*. To this end, wild type mouse tibia were fixed, decalcified, and sectioned. Sections were stained for the osteoclast marker tartrate-resistant acid phosphatase (TRAP), immunostained for the osteoblast marker Cbfa1, immunostained for neurofibromin, or stained following incubation with IgG as control. As shown in **figure 2 (appended item 4)** neurofibromin expression is seen in osteoblasts, in chondrocytes, and in megakaryocytes in the marrow. Bone lining cells, irrespective of their Cbfa1 status (identifying differentiating osteoblasts) all express neurofibromin.

For *in vitro* assays we have purified primary osteoblasts from the NF1ob/ob animals, with the col1-Cre and NF1flox/flox animals described above (*Task 1*) as controls, using standard protocols (6). Frozen stocks of these cells can now be used as a source of primary osteoblasts to determine the effect of NF1 inactivation on osteoblast differentiation *in vitro*. In preparation for these experiments, and as an alternate approach, we used the pre-osteoblast MC3T3e1 cell line (7). Using MC3T3-E1 we have determined conditions and procedures for the differentiation time course, RNA isolation, as well as the QPCR conditions for measuring neurofibromin and osteoblast marker gene expression in these cells. **Figures 3 (appended item 5)** shows Nf1 expression in

early differentiation of MC3T3-E1 cells. **Figure 4 (appended item 5)** shows long-term differentiation including matrix mineralization with Nf1 expression, osteocalcin (Ocn) and osteopontin (Opn) expression. We find that Nf1 expression peaks along with Ocn expression and begins at the onset of mineralization.

Until we had primary cells in which Nf1 gene is inactivated, we turned to RNA interference technology (RNAi) to produce Nf1 deficient cells from osteoblast precursor cells. We use commercially available Nf1 siRNA expressing lentiviral particles (MISSION lentiviral transduction particles, Sigma and The RNAi Consortium). The lentiviral particles are available in groups of five clones with different target sequences to rule out off-target effects. Positive (GFP) and negative control transduction particles are also available. We employed these particles to assess the effects of lentiviral transduction on osteoblast differentiation, gene expression, and function. Cells were transduced at several multiplicity of infection. Cells were then selected, by culture with puromycin, and Nf1 expression monitored by QPCR as described above and in **figures 3** and **4**. As shown in **figure 5 (appended item 6)** with one of the five target sequence clones we can achieve moderate silencing (>50%). We anticipate that some of the clones targeting other sequences will have a greater effect on message levels and the effect should be additive with a combination of two, three, or four siRNA vectors. Preparation of osteoblast cell pools expressing various levels of Nf1 will allow us to monitor differentiation and phenotype in the presence of various levels of Nf1 and to correlate effects with gene dose, an aspect which is impossible to test with Nf1-/- , Nf1+/-, or Nf1ob/ob animal cells.

Task 3. **Characterize osteoclast formation and function from Nf1+/- mouse cells in culture:** Due to the limitations outlined above (*Task 1*) we have been unable to test primary osteoclast precursors from NF1 +/- mice. We have again turned to RNA interference to test various levels of Nf1 knockdown on osteoclast differentiation and function. We have begun with an osteoclast forming cell line RAW264.7, which we have extensively characterized (8). To this end, and following significant optimization of the protocol, we have determined conditions for lentiviral transduction with subsequent osteoclast formation assay. Following the optimized protocol, RAW264.7 cells were plated at low density and transduced with GFP cassette-containing lentiviral particles at an apparent MOI ≈ 20:1. Cells were selected for 4 days with puromycin, with or without RANKL at 20ng/ml. As shown in **figure 6 (appended item 6)**, GFP producing RAW264.7 cells are produced using this protocol and, with RANKL treatment, multinuclear GFP-positive osteoclast cells are produced. In our experience RAW264.7 cells expressing this morphology express most osteoclast genes and typically behave as genuine osteoclasts (8). Untransduced RAW264.7 cells formed osteoclasts in the expected manner and, in the absence of transduction, no cells survive puromycin treatment (data not shown).

Following testing of Nf1 silencing in RAW264.7 cells by QPCR, siRNA expressing particles found to be effective in Nf1 silencing will be used to infect primary mouse bone marrow macrophage using a similar protocol which includes puromycin selection. Nf1 expression will be measured in transduced cells which will then be differentiated to osteoclasts, which will be assayed for resorptive function. This approach, in contrast to primary cells from Nf1+/- or-/- cells, allows us to correlate degree of Nf1 expression with any phenotype observed.

Task 4. **Characterize fracture repair in Nf1+/- mice:** While awaiting test animals we have established the fracture model for wild type mice. Our collaborator, Louis Gerstenfeld from Boston University Medical School, instructed us in the procedures for the mouse fracture model. In addition, we purified RNA over the time course of fracture healing in wild type animals and measured neurofibromin expression by QPCR (using QPCR conditions as determined in **figure 3**) establishing the time course of neurofibromin expression over fracture healing. As shown in **figure 7 (appended item 7)** neurofibromin mRNA steady-state levels increased in early fracture healing, consistent with the endochondral/primary bone formation stage in the previously described time course (9).

To characterize the cell types expressing neurofibromin in wild type bone fracture healing, we have fixed, decalcified, and sectioned samples of wild type mouse bone fracture over the course of healing. Using Immuno detection of neurofibromin was detected on day 7 at sites of primary bone formation and in bone lining cells in the callus (**figure 8; appended item 8**). Little neurofibromin expression was seen in the cartilagenous fracture callus or in marrow cells. We made an interesting observation in the process of optimizing conditions for immunostaining of Nf1 in mouse bone. Optimization was done on bone samples from a mouse model of rheumatoid arthritis (RA). In this model we find that Nf1 expression is absent in the region of osteolytic joint destruction. The lack of Nf1 expression corresponds to a region which also lacks osteoblast-mediated repair. Nf1 expression is however seen at distant sites in osteoblasts and bone-lining cells. While not directly related to the aims of this project, this observation may provide insight into the pathogenesis of RA and will be pursued in collaboration with RA researchers.

Task 5. **Characterize the bone cells and molecules present in samples from Orthopedic NF1 patients:** the protocol was approved by the Beth Israel Deaconess Medical Center (BIDMC) Committee on Clinical Investigations (CCI) on 27, November 2007 and the Children's Hospital Boston CCI on 10, September 2007. The protocol was reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable Federal, DOD, U.S. Army, and USAMRMC human subjects protection requirements. The protocol was approved by USAMRMC HRPO on December 6, 2007.

Under these approved protocols we have established a bank of samples which, to date, includes samples from 7 patients. The protocols were initially approved for sample collection from 12 NF1 patients and 3-5 control patients over three years. In their consent forms, all patients, thus far, have agreed to allow storage and use of their tissue for future research related to NF1. All but two have also agreed to allow storage of the tissue for future research *not* related to NF1. All tissues are stored in the PI's laboratory and demographics forms are stored under lock and key in the office of the PI. Signed consent forms, copies of the demographics forms, and the ID assignment log are kept in the Orthopedic Clinical Study coordinator's office at Children's Hospital in a locked file.

Of the seven patient tissue samples currently in hand, four samples are from NF1 patients and three samples are from control patients. Three control and three NF1 samples are from scoliosis surgeries and one sample is from a tibial pseudoarthrosis patient. All patient samples include three or more bone tissue fragments and some include over 12 individual bone fragments. All samples have been fixed and decalcified. Samples from five patients have been imbedded in paraffin and sectioned while the remaining samples are currently being processed. Selected sections have been stained with H&E stain for general histological analysis or histochemically stained for tartrate-resistant acid phosphatase (TRAP) in order to identify osteoclast cells. Tissue blocks and additional section slides are stored in the PI's laboratory.

As shown in **figure 9 (appended item 9)** for representative samples H&E staining identifies normal gross bone histology in NF1 samples **(figure 9C.** and **9D.)**. TRAP stained osteoclasts **(figure 9)** are seen in resorption lacunae in control bone tissue samples **(figure 9A.** and **9B.)** while osteoclasts in NF1 patient bone samples tend to be smaller **(figure 9 E.** and **F.)**. NF1 osteoclasts are found to be on the order of 1-3 nuclei per osteoclast vs. 3-7 for control patient bone tissue. NF1 osteoclasts also appear to express less of the TRAP enzymatic activity **(figure 9)**. Any conclusions about NF1 bone tissue or cell abnormalities require bone histomorphometric analysis of additional samples.

KEY RESEARCH ACCOMPLISHMENTS

Results

1. Establish a breeding colony of col1-Cre;Nf1flox/flox (NF1ob/ob) mice for conditional, lineage-specific, knockout of the neurofibromin gene in osteoblasts. Nf1flox/flox mice for knockout of the neurofibromin gene using exogenous Cre recombinase in osteoclast precursors.

2. Establishment and optimization of assay conditions (primer sequences, primer efficiency, amplification conditions, etc.) for real-time RT-PCR amplification of mouse neurofibromin and osteoblast marker genes (osteopontin and osteocalcin).

3. Testing lentiviral delivery and siRNA-mediated Nf1 gene silencing in osteoblast (MC3T3-E1) and osteoclast (RAW264.7) cells.

4. Time course of neurofibromin mRNA steady-state expression levels over osteoblastic differentiation of MC3T3-E1 cells.

5. Establishment of the mouse fracture repair model and preparation of RNA and fixed bone samples for days 5, 7, 10, and 14 for wild type animals.

6. Measurement of neurofibromin mRNA steady-state expression levels over the course of bone fracture healing in a mouse model.

7. Immunohistochemical detection of neurofibromin in normal mouse bone and in a mouse model of fracture healing.

8. Establish a bank of bone tissue samples and slides from NF1 patients and control patients with which to compare to mouse samples at the tissue and cellular levels.

REPORTABLE OUTCOMES

Publications

Manuscripts:

1. O'Sullivan RP, Crotti TN, Walsh NC, Barnes GL, Gerstenfeld LC, and McHugh KP. Neurofibromin (NF1) Expression in Differentiating Osteoblasts In Vitro and in Fracture Healing. manuscript in preparation.

Abstracts:

1. Crotti TN, Walsh NC, Barnes GL, Gerstenfeld LC, McHugh KP. Neurofibromin expression in mouse bone and in fracture healing. The Children's Tumor Foundation, International Consortium for the Molecular and Cellular Biology of NF1, NF2, and Schwannomatosis Aspen, Colorado, 2006. (**appended Item 1**)

2. Crotti TN, Walsh NC, Barnes GL, Gerstenfeld LC, McHugh KP. Neurofibromin expression in bone fracture healing. Journal of Bone and Mineral Research 2006; 21:S429. (**appended Item 2**)

Personnel receiving pay from the research effort:
Kevin P. McHugh, Ph.D.
Merrilee Flannery
Zhenxin Shen, Ph.D.

CONCLUSIONS

Cells expressing Nf1 protein *in vivo* have been identified by immunohistochemistry (IHC). IHC for Cbfa-1 was used to identify osteoblast-lineage cells and TRAP staining identified osteoclast-like cells. Nf1-positive cells were abundant within the primary spongiosa. Chondrocytes and megakaryocytes were also Nf1-positive. TRAP positive osteoclast-like cells on the bone surface were weakly positive for Nf1. Bone-adherent osteoblast lineage cells, irrespective of Cbfa-1 expression, uniformly expressed Nf1. Therefore, bone-forming osteoblasts, as well as quiescent bone-lining cells, express Nf1 in physiological conditions. These osteoblast-lineage cells mediate access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism. We have also measured Nf1 expression in MC3T3-E1 cells over the course of differentiation. Using RT-QPCR, we find that Nf1 mRNA expression is initially low and increases over the course of early osteoblastic differentiation with a peak corresponding to the onset of osteocalcin expression and matrix mineralization by MC3T3-E1 cells.

We can show >50% Nf1 gene silencing using siRNA lentiviral vectors in osteoblasts *in vitro* and have optimized lenti-mediated transduction of RAW264.7 cells with subsequent osteoclast formation. The availability of these cells will allow us to correlate cell function with the extent of knockdown. In contrast to primary cells, which have normal expression (wild type), partial expression (+/-), or no expression (-/-), we anticipate that we will be able to isolate cells with several intermediate levels of Nf1 expression. This is due to different multiplicity of infection used and the knock down efficiencies of different siRNA constructs.

We have also examined the expression of neurofibromin across the time course of bone fracture healing in a mouse model of fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification, with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation (9). Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. In the mouse model of fracture healing, neurofibromin expression was increased by four-fold 5 days post-fracture, with expression declining by day 14 and reduced to background levels by 21 days post-fracture. This time course of neurofibromin mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. Elevated rates of bone resorption are typically observed in the second to fourth weeks post-fracture, during primary and secondary bone formation. Therefore, osteoclastic bone resorption coincides with the decline in neurofibromin expression. Immunohistochemical staining of healing fractures in wild type animals indicates neurofibromin expression in areas of primary bone formation. Taken together these observations indicate that neurofibromin expression is associated with bone-adherent osteoblast-lineage cells with minimal expression in osteoclasts. In addition neurofibromin expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing.

Bone tissue samples from NF1 patients, and controls indicate possible differences in osteoclast morphology. NF1 patient osteoclasts appear smaller and display low TRAP activity, however these results require bone histomorphometric analysis of additional samples. It is possible that an osteoclast defect is secondary to an osteoblast defect since osteoblasts support osteoclast differentiation in their early stages of differentiation. The patient samples are also an invaluable resource for future studies of NF1 bone tissue.

Little is known of the cellular or molecular characteristics of NF1 bone tissue that lead to skeletal manifestations of the disease. In this respect any and all observations and information gleaned are important to the basic science of NF1. Osteoblastic and osteoclastic defects have both been described in recent scientific literature. The important functional defect is still unclear and our results indicate it is likely primarily an osteoblast defect. While we are still quite a distance from clinical practice, this work brings us closer to understanding the disease and finding effective treatments.

REFERENCES

1. Stevenson, D.A., Zhou, H., Ashrafi, S., Messiaen, L.M., Carey, J.C., D'Astous, J.L., Santora, S.D., and Viskochil, D.H. 2006. Double inactivation of NF1 in tibial pseudarthrosis. *Am J Hum Genet* 79:143-148.
2. Elefteriou, F., Benson, M.D., Sowa, H., Starbuck, M., Liu, X., Ron, D., Parada, L.F., and Karsenty, G. 2006. ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae. *Cell Metab* 4:441-451.
3. Aliprantis, A.O., Walsh, N.C., McHugh, K.P., Gravallese, E., and Glimcher, L.H. 2007. Conditional mutagenesis reveals that NFATc1 is required for osteoclast differentiation in vivo. *Annals of the Rheumatic Diseases* 66:A6-A7.
4. Crotti, T.N., Sharma, S.M., Fleming, J.D., Flannery, M., Ostrowski, M.C., Goldring, S.R., and McHugh, K.P. 2008. PU.1 and NFATc1 mediate osteoclastic induction of the mouse β3 integrin promoter. *Journal of Cellular Physiology* in press.
5. Crotti, T.N., Flannery, M., Walsh, N.C., Fleming, J.D., Goldring, S.R., and McHugh, K.P. 2006. NFATc1 regulation of the human beta3 integrin promoter in osteoclast differentiation. *Gene* 372:92-102.
6. Helfrich, M.H., and Ralston, S.H., editors. 2003. *Bone research protocols*. Totowa, N.J.: Humana Press.
7. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P.H., and Franceschi, R.T. 1999. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. *J Bone Miner Res* 14:893-903.
8. Cuetara, B.L., Crotti, T.N., O'Donoghue, A.J., and McHugh, K.P. 2006. Cloning and characterization of osteoclast precursors from the RAW264.7 cell line. *In Vitro Cell Dev Biol Anim* 42:182-188.
9. Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., and Einhorn, T.A. 2003. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* 88:873-884.
10. Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.

APPENDICES

Pages

Appended item 1.

Crotti TN, Walsh NC, Barnes GL, Gerstenfeld LC, McHugh KP. Neurofibromin expression in mouse bone and in fracture healing. The Children's Tumor Foundation, International Consortium for the Molecular and Cellular Biology of NF1, NF2, and Schwannomatosis, Aspen, Colorado, 2006.

A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including alterations in bone size and shape, the presence of scoliosis, and a tendency to develop pseudoarthrosis. These skeletal manifestations of NF1 are further compounded by generalized skeletal osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. Normal bone remodeling and healing require the concerted actions of bone forming osteoblasts and bone resorbing osteoclasts. However, the cell type and pathway by which neurofibromin haploinsufficiency leads to dysregulation of normal bone remodeling and healing are unknown.

To better understand the function of neurofibromin in normal bone cell physiology and fracture healing, we used immunohistochemistry (IHC) to identify cells expressing neurofibromin protein in vivo. In addition, IHC for Cbfa-1 was used to identify osteoblast-lineage cells and histochemical staining for tartrate-resistant acid phosphatase (TRAP) identified osteoclasts. In tibiae isolated from young C57Bl6/J mice, neurofibromin-positive cells were abundant within the primary spongiosa, located below the growth plate, which is an active site for resorption of calcified cartilage and formation of trabecular bone. TRAP positive osteoclasts throughout the bone were weakly positive for neurofibromin. Neurofibromin was uniformly expressed by bone-adherent osteoblast lineage cells regardless of their status for Cbfa-1 staining. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express neurofibromin in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism.

We next examined the expression of neurofibromin across the time course of bone fracture healing using quantitative RT-PCR with RNA samples collected from a mouse model of fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification, with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation. Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. In the mouse model of fracture healing neurofibromin mRNA expression was increased by four-fold 5 days post-fracture, with expression declining by day 14 and reduced to background levels by 21 days post-fracture. This time course of neurofibromin mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. Elevated rates of bone resorption are typically observed in the second to fourth weeks post-fracture, during primary and secondary bone formation. Therefore, osteoclastic bone resorption coincides with the decline in neurofibromin expression. Taken together these observations indicate that neurofibromin expression is associated with bone-adherent osteoblast-lineage cells with minimal expression in osteoclasts. In addition neurofibromin expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing.

In future experiments characterization of the cellular and molecular events associated with bone healing in the *Nf1*+/- mouse fracture model will provide a model in which to test new strategies aimed at improving bone quality and healing, leading to improved outcomes and reducing the negative economic, social, and physical impact of this disorder in the primarily pediatric orthopaedic NF1 population.

Appended item 2.

Crotti TN, Walsh NC, Barnes GL, Gerstenfeld LC, McHugh KP. Neurofibromin expression in bone fracture healing. 28th Annual meeting of the American Society for Bone and Mineral Research, Philadelphia, Pennsylvania, 2006. Journal of Bone and Mineral Research 21:S429.

A large proportion of patients with Neurofibromatosis Type 1 display skeletal abnormalities including scoliosis and pseudoarthrosis, which are compounded by osteoporosis and poor bone healing. Corrective orthopaedic intervention often fails, necessitating multiple revision surgeries followed by prolonged recovery periods. The cell types and pathway by which neurofibromin haploinsufficiency (*Nf1* +/-) leads to dysregulation of bone remodeling and healing are unknown. The aim of this study is to identify the cell types expressing *Nf1* in normal bone cell physiology and fracture healing.

Tibiae were isolated from young C57Bl6/J mice and the cells expressing *Nf1* protein *in vivo* were identified by immunohistochemistry (IHC). IHC for Cbfa-1 was used to identify osteoblast-lineage cells and TRAP staining identified osteoclast-like cells. *Nf1*-positive cells were abundant within the primary spongiosa. TRAP positive osteoclast-like cells on the bone surface were weakly positive for *Nf1*. Bone-adherent osteoblast lineage cells, irrespective of Cbfa-1 expression, uniformly expressed *Nf1*. This observation indicates that bone-forming osteoblasts, as well as quiescent bone-lining cells, express *Nf1* in physiological conditions. Both of these osteoblast-lineage cells function in mediating access of osteoclasts to bone surface, which is thought to regulate the rate of initiation of the bone-modeling unit and thereby determine the rate of bone metabolism. To verify gene expression of *Nf1* in bone lineage cells we measured *Nf1* expression in MC3T3-E1 cells over the course of differentiation. Using RT-QPCR, we find that *Nf1* mRNA expression is initially low and increases over the course of early osteoblastic differentiation of MC3T3 cells.

We next examined the expression of *Nf1* in a mouse model of fracture healing. mRNA expression was measured by RT-QPCR in samples taken across the time course of bone fracture healing. Cellular events in fracture healing recapitulate those observed in endochondral ossification, with chondrocyte formation of a cartilage model, which is replaced by woven bone in primary bone formation. Woven bone is subsequently remodeled to lamellar bone, by coupled remodeling in secondary bone formation. We find that *Nf1* mRNA expression was increased by four-fold 5 days post-fracture, with expression declining by day 14 and reduced to background levels by 21 days post-fracture. This time course of *Nf1* mRNA expression lags the immediate inflammatory response and closely parallels the endochondral-like mineralized callus formation. *Nf1* expression is induced during the formation of the mineralized callus in the endochondral-like formation stage of bone fracture healing.

Appended item 3

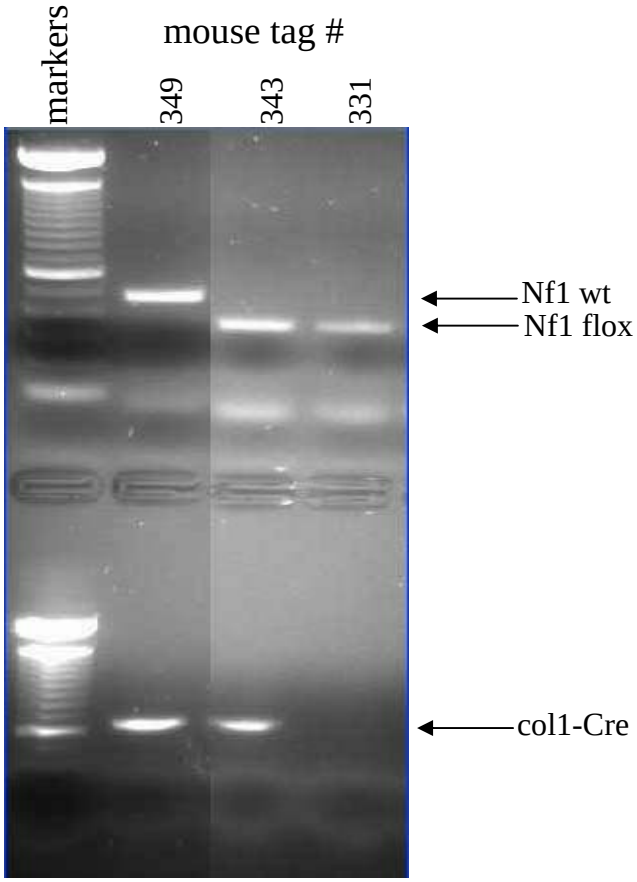

Figure 1. PCR genotyping of Nf1ob/ob animals and controls. 1% agarose gel showing genotypes for representative animals indicating homozygosity for wild type Nf1 gene, the LoxP site flanked Nf1 gene, and for for presence or absence of the col1-Cre transgene. Individual animals are: Nf1wt/wt;col1-Cre (#349; col1-Cre), Nf1flox/flox;col1-Cre (#343; Nf1ob/ob), and Nf1flox/flox (#331). An intervening lane in the original gel has been omitted.

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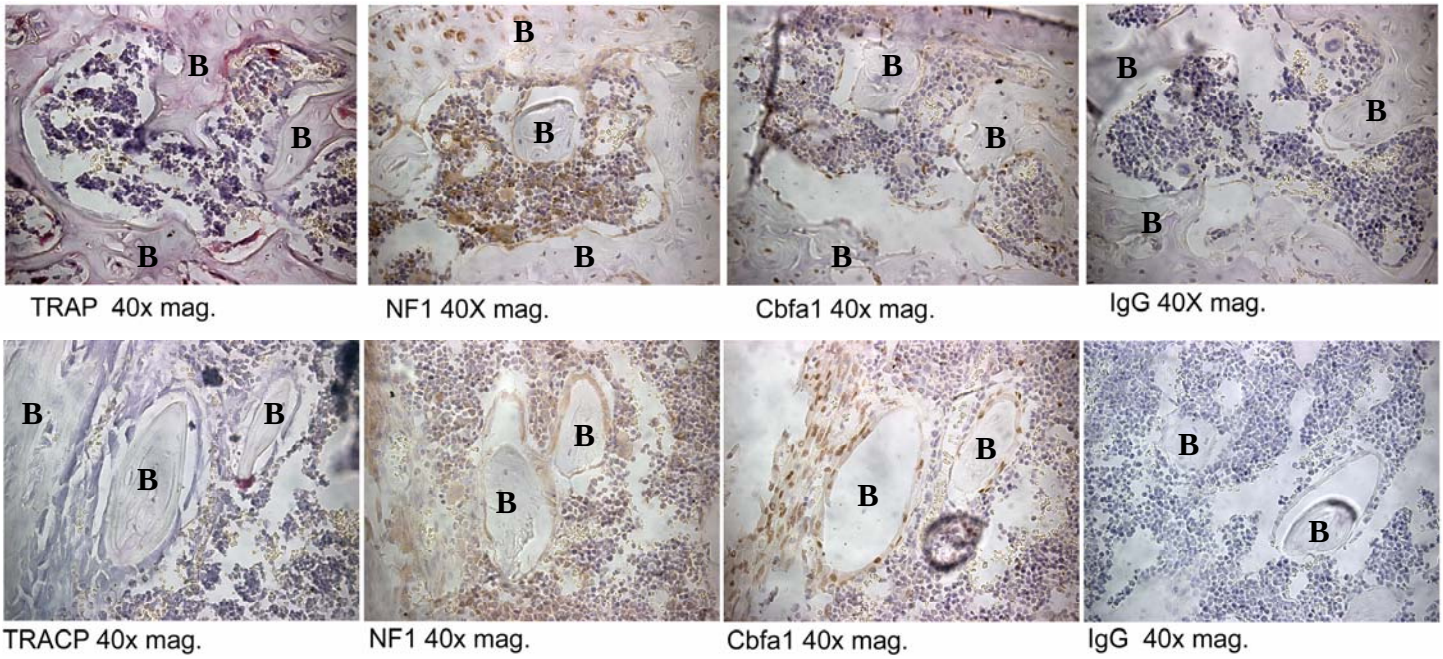

Figure 2. Immunohistochemical detection of neurofibromin in osteoblast cells *in vivo*. Tibiae from wild type mice were fixed in 4% paraformaldehyde (PFA) and decalcified with EDTA (0.5M for 3 days). Serial sections (5mm) were prepared and probed with antibodies against neurofibromin (sc-67; SantaCruz) or the osteoblast marker Cbfa1 (sc-10758; SantaCruz), or osteoclasts were stained for TRAP (red stain). After washing, antibody was detected with HRP-conjugated secondary antibody and counter stained with hematoxylin. Controls with no primary or no secondary antibody are clean (IgG). In the figure, bone tissue is identified by "B". All bone-adherent cells (osteoblasts and lining cells) stained positive for neurofibromin. The osteoblast transcription factor Cbfa1 displays nuclear distribution in the differentiating sub-population of osteoblasts.

Appended item 5

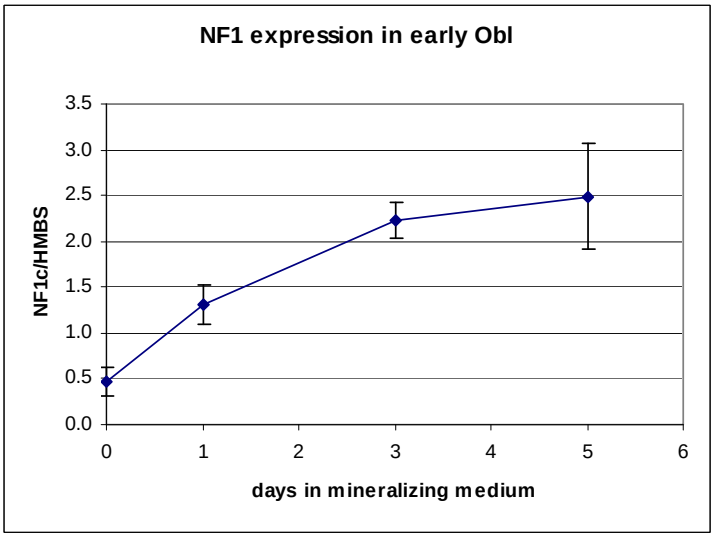

Figure 3. Gene expression in osteoblastic differentiation of MC3T3-E1 cells. Cells were plated and allowed to grow to confluence when medium was changed to mineralizing medium containing β-glycerolphosphate and ascorbic acid (= day 0). RNA was purified at various time points up to 5 days and reverse transcribed. QPCR was performed for neurofibromin and HMBS as control. Expression relative to HMBS was determined using the $2^{-\Delta\Delta Ct}$ method (10). NF1 expression, relative to HMBS, was shown to increase within the first few days of culture under differentiation conditions.

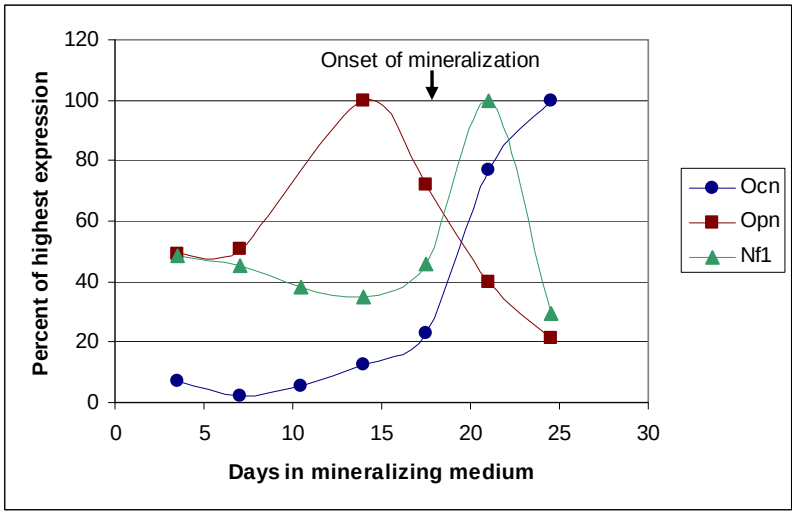

Figure 4. In longer term mineralization experiments RNA was purified at various time points up to 25 days and reverse transcribed. QPCR was performed for the osteoblast marker genes, osteocalcin (Ocn) and osteopontin (Opn), and neurofibromin (Nf1), with HMBS as control. Relative expression was plotted as the percent of highest expression. Matrix mineralization was monitored by Von-kossa (mineral) staining of parallel cultures. In long-term experiments, Nf1 expression peaks with Ocn expression and begins at the onset of mineralization (arrow).

Appended item 6

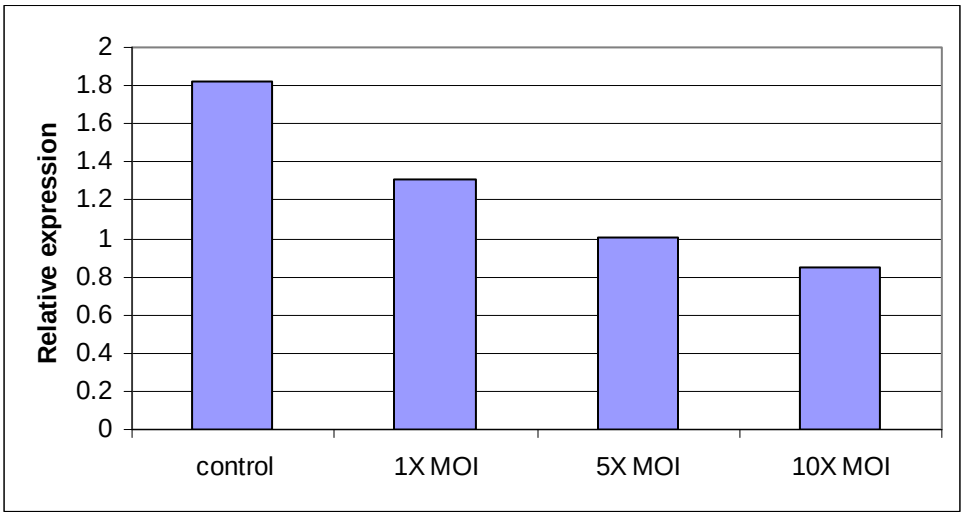

Figure 5. Nf1 silencing in osteoblasts with lenti-siRNA transduction. MC3T3-E1 cells were plated in 12 well plates and transduced with increasing multiplicity of infection (MOI) with a single siRNA construct. Following puromycin selection, RNA was purified and Nf1 mRNA expression was quantified by QPCR. MOI is relative since the true titer of infectious particles is not known. Gene silencing of approximately 54% was achieved at the highest MOI tested with this construct.

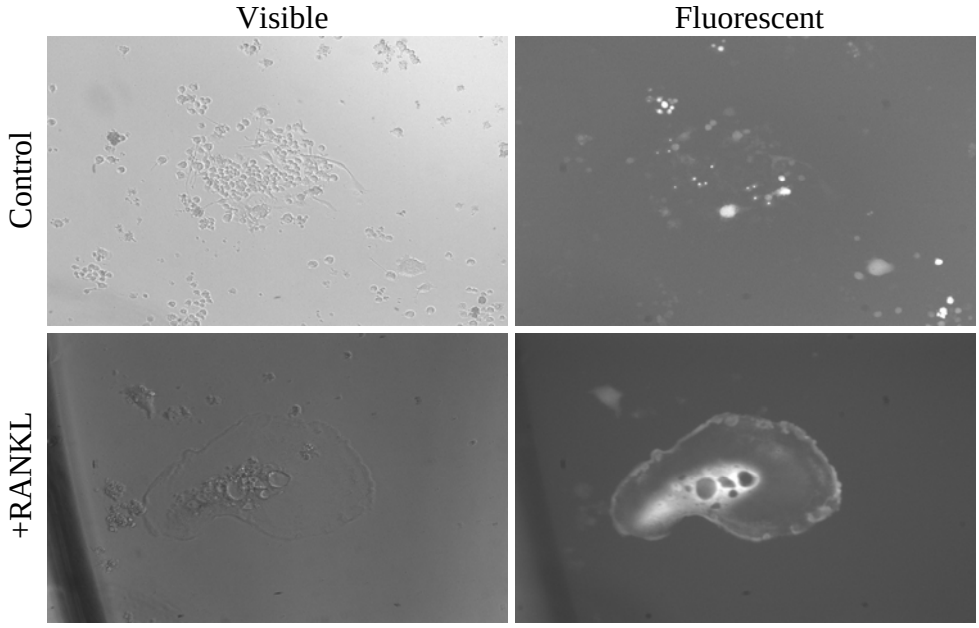

Figure 6. Lenti-GFP transduction of RAW264.7 cells with subsequent osteoclast formation. RAW264.7 cells were plated at low density (5 X 10^3/cm^2) and allowed to adhere over night. Lentiviral particles were added at 10μl/cm^2 from stock at 1 X 10^7/ml (apparent MOI ≈ 20:1). After 24 hours, medium was changed and transduced cells were selected for 4 days with puromycin, with or without RANKL at 20ng/ml.

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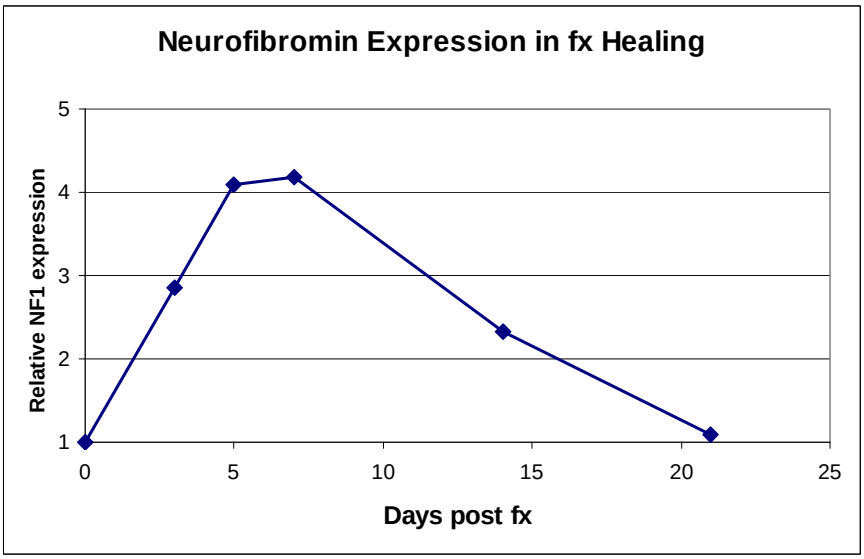

Figure 7. Quantitative RT- PCR for neurofibromin in the mouse model of fracture healing. RNA was purified from fracture calluses, on the days post-fracture indicated, and neurofibromin message steady-state levels were measured by real-time RT-QPCR using Sybr green dye incorporation. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method as described for **figure 2**. Neurofibromin mRNA steady-state levels were seen to increase in early fracture healing, consistent with the endochondral/primary bone formation stage described (9).

Appended item 8

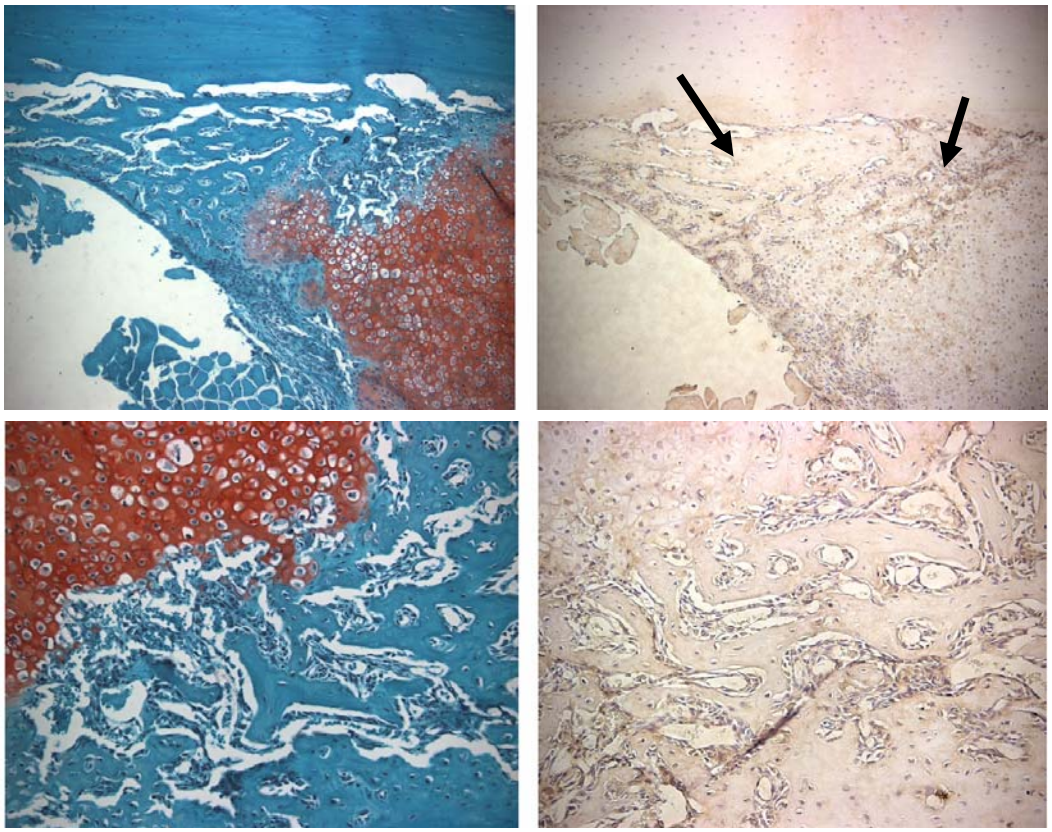

Figure 8. Immunohistochemical detection of neurofibromin in fracture healing of mouse bones. Femora of wild type mice (C57Bl/6) were fractured and after 5, 7, 10 or 14 days bones were harvested. Bones were fixed in 4% paraformaldehyde and decalcified with EDTA (0.5M for 8 days). Serial sections (5mm) were prepared and probed with antibodies against neurofibromin (sc-67; SantaCruz) as described in **figure 2**. After washing, antibody was detected with HRP conjugated secondary antibody and counterstained with hematoxilin (right panels). Adjacent sections were stained with safarin O and fast green (left panels). Using safarin O and fast green cartilage is stained bright red and bone is stained light blue. Primary bone formation occurs with the resorption of cartilage while secondary bone formation and remodeling to lamellar bone occurs from the edge of the fracture callus. In the day 7 samples shown, neurofibromin expression was seen at sites of primary bone formation (arrows) and in bone lining cells. Little neurofibromin expression was seen in the cartilagenous fracture callus. Due to the poor preservation of cellular morphology of bone-adherent cells we were unable to identify osteoclasts or chondroclasts in these samples.

Appended item 9

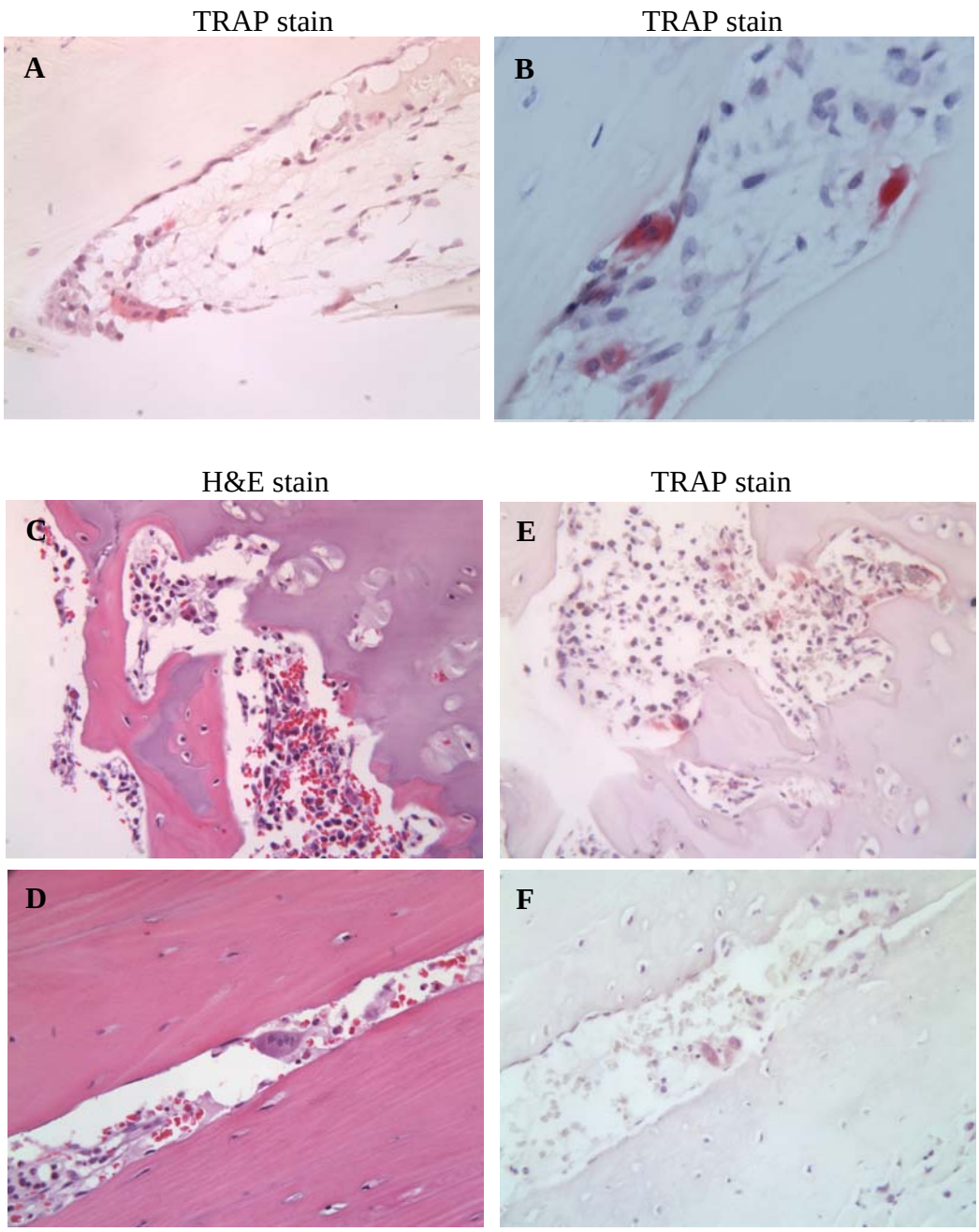

Figure 9. Initial histological examination of bone tissue from control and NF1 patients. Bone tissue sections were prepared essentially as described for mouse samples described in **figure 6.** Sections of control bone sample from patient #OG2-001 stained for tartrate-resistant acid phosphatase (TRAP) at 200X **A.** and at 400X **B.** Panels **C.** and **D** are sections of NF1 bone tissue from patient OG2-003 stained with hematoxylin and eosin stain (H&E) viewed at 200X. H&E stains bone pink, nuclei blue, and red blood cells bright red. Panels **E.** and **F.** show sections from patient OG2-003, near to sections seen in **C.** and **D.**, stained for TRAP and viewed at 200X. Sections **C.** and **E.** are taken near facet joints of the spine and contain growth plate-like and cartilaginous structures.